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## Reprogrammed quiescent B cells provide an effective cellular therapy against chronic experimental autoimmune encephalomyelitis

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### Abstract

Activated B cells can regulate immunity, and have been envisaged as potential cell-based therapy for treating autoimmune diseases. However, activated human B cells can also propagate immune responses, and the effects resulting from their infusion into patients cannot be predicted. This led us to consider resting B cells, which in contrast are poorly immunogenic, as an alternative cellular platform for the suppression of unwanted immunity. Here, we report that resting B cells can be directly engineered to express antigens in a remarkably simple, rapid, and effective way with lentiviral vectors. Notably, this neither required nor induced activation of the B cells. With that approach we were able to produce reprogrammed resting B cells that inhibited antigen-specific CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and B cells upon adoptive transfer in mice. Furthermore, resting B cells engineered to ectopically express myelin oligodendrocyte glycoprotein antigen protected recipient mice from severe disability and demyelination in experimental autoimmune encephalomyelitis, and even induced complete remission from disease in mice lacking functional

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natural regulatory T cells, which otherwise developed a chronic paralysis. In conclusion, our study introduces reprogrammed quiescent B cells as a novel tool for suppressing undesirable immunity.

## Keywords

B cells; autoimmunity; gene therapy; lentiviral vector; cellular therapy

## INTRODUCTION

Recent studies have identified novel roles of activated B cells in the inhibition of immunity [1-3]. These suppressive functions of B cells could be of interest to therapeutically block unwanted immune responses [1-3].

To suppress ongoing autoimmune diseases in mice B cells must be activated via Toll-like-receptors (TLR), B cell receptor for antigen (BCR), and CD40, which result in sustained production of the immunosuppressive cytokine interleukin(IL)-10 [2, 4]. These activation pathways may therefore be useful to produce therapeutic regulatory B cells. In keeping with this, B cells activated with selected TLR agonists *in vitro* effectively suppressed various autoimmune diseases upon adoptive transfer in recipient mice [5, 6]. The protective function of these activated B cells depended on presentation of disease-relevant autoantigens via major histocompatibility complex (MHC)-II molecules [5]. Importantly, activated B cells can also exert stimulatory activities, and the mechanisms distinguishing their pro- from anti-inflammatory functions have not been fully identified [7-10]. Thus, currently it is not possible to predict whether an *ex vivo* activated human B cell will mediate suppressive or stimulatory functions upon administration into patients. Consequently, the utilization of activated B cells may appear not safe enough for clinical application in humans. On the other hand, resting B cells could provide a safer platform for adoptive therapies: they express low levels of costimulatory molecules and they do not secrete antibodies [11]. Resting B cells can induce tolerance in naive T cells, but are less effective than some activated B cells in inhibiting ongoing immune reactions [11-13].

We therefore reasoned that the ideal therapeutic B cell should combine the weak immunogenicity of resting B cells with the effective suppressive functions of some activated B cells. In order to construct such “resting regulatory B cells”, we established a novel gene therapy protocol to genetically reprogram resting B cells while keeping them in a quiescent state. This enabled us to produce resting B cells presenting antigen to reactive T cells, and secreting the cytokine IL-10, which are two key features of suppressive activated B cells [1-3, 5]. We demonstrate that such reprogrammed resting B cells can suppress unwanted immune reactions mediated by CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and B cells upon adoptive transfer in recipient mice. These B cells protected recipient mice from chronic disability and demyelination in experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (MS). Altogether, our study introduces genetically reprogrammed resting B cells as a new vehicle for the inhibition of immunity.

## RESULTS

### Effective engineering of unstimulated quiescent B cells by lentiviral transduction

We established a lentiviral-based protocol to genetically reprogram quiescent B cells. Plain centrifugation of resting B cells with lentiviral particles for 75 minutes was sufficient to obtain high transduction efficiency (Fig. 1A).

B cells express pathogen recognition receptors for viral components, whose triggering can lead to up-regulation of co-stimulatory molecules, and acquisition of increased immunostimulatory properties [10, 14]. Lentiviral transduction did not result in statistically significant alteration of the expression levels of MHC-II, CD40, CD44, CD69, CD80, CD86, and IL-6 by the genetically modified B cells kept *in vitro* for 18 hours compared to naive B cells, while these molecules were significantly up-regulated on B cells activated with the TLR-4 agonist lipopolysaccharides (LPS) (Figs. 1B-D). Similarly, transduced B cells maintained phenotypic features of resting B cells upon adoptive transfer in recipient mice (Fig. 1E). From these results, we conclude that lentiviral vectors allow the genetic engineering of quiescent B cells while maintaining them in a resting state.

### Engineering quiescent B cells to present antigen and secrete IL-10 by lentiviral transduction

Vectors encoding for the mouse *Il-10* gene (pDBR-IL-10), or for full-length mouse myelin oligodendrocyte glycoprotein (MOG) gene (pDBR-MOG) were used to generate resting B cells secreting IL-10 and/ or expressing MOG. Culture supernatants from B cells transduced with either pDBR-IL-10 (B-IL-10 cells) or both pDBR-MOG and pDBR-IL-10 (B-MOG-IL-10 cells) contained around 8 ng/ml IL-10, while this cytokine was undetectable in supernatants from B cells transduced with lentiviral particles encoding for the human *Cd4* gene (*hCd4*; pDBR-hCD4; B-hCD4 cells) (Fig. 2A). This B cell-derived IL-10 was functional since it inhibited TNF- $\alpha$  production by CpG-activated macrophages similarly to recombinant IL-10 (Fig. 2B).

To assess the possibility of designing B cells presenting relevant antigen to CD4<sup>+</sup> T cells, resting B cells were transduced with pDBR-MOG (B-MOG cells), and cultured with the MOG-reactive T cell hybridoma P32.2, which secretes IL-2 upon stimulation with MOG-MHC-II complexes [15]. B-MOG and B-MOG-IL-10 cells were both able to elicit IL-2 production by P32.2 (Fig. 2C). These B cells presented MOG-derived epitopes to P32.2 via MHC-II because the IL-2 response was abolished in co-cultures supplemented with a blocking anti-MHC-II antibody (Fig. 2D).

### Reprogrammed quiescent B cells provide protection from chronic EAE

We evaluated the suppressive capacity of reprogrammed quiescent B cells in EAE, a T cell-mediated autoimmune disease that leads to development of inflammatory demyelinating lesions in central nervous system (CNS), and mimics features of MS.

Administration of B-MOG-IL-10 cells to recipient mice on days -6 and -2 prior to immunization resulted in reduced disease severity and improved recovery from EAE, compared to mice treated with control B-mock cells (i.e. B cells transduced with an “empty” pDBR vector), or to untreated mice (Figs. 3A,B). Under this treatment, B-MOG cells were as protective as B-MOG-IL-10 cells, while B-IL-10 cells had no significant therapeutic effect, suggesting that the protective effect was independent of IL-10 (Fig. 3C). Indeed, B-MOG cells prepared from IL-10-deficient B cells led to similar amelioration of disease as B-MOG cells engineered from wild-type B cells (Fig. 3D). Thus, resting B cells expressing MOG provided protection from EAE, but IL-10 secretion did not increase their beneficial effect. As a consequence of the latter finding, we performed all subsequent experiments with B-MOG cells.

We then tested the efficacy of this therapeutic approach in an experimental condition normally leading to severe chronic demyelinating disease. Natural regulatory CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells (Treg) are critical for recovery from EAE, and they are impaired in patients with MS [16, 17]. As expected, administration of an anti-CD25 antibody to mice

prior to immunization with MOG(35-55), resulting in impairment of Treg numbers and functions, induced a severe form of chronic EAE (Fig. 3E). Remarkably, adoptive therapy with B-MOG cells protected mice from this chronic form of EAE, allowing a full recovery from disease by day 35 after immunization (Fig. 3E). Thus, B-MOG cells could replace Tregs for disease resolution. In contrast, B-MOG cells could not reduce maximal disease score in anti-CD25-treated mice, indicating that Tregs provided a non-redundant mechanism limiting the severity of acute disease.

### Reprogrammed quiescent B cells suppress T cell- and B cell-mediated immunity

In order to identify how B-MOG cells facilitate remission from EAE, we monitored encephalitogenic T and B cell responses at different time points after EAE induction in mice treated or not with B-MOG cells.

Autoreactive T helper cells can be identified and enumerated by measuring upregulation of CD154 (CD40L) on CD4<sup>+</sup> T cells re-stimulated with antigen *ex vivo* (Fig. 4A) [18]. Following immunization with MOG(35-55), MOG-reactive CD4<sup>+</sup> T helper cells transiently accumulated in draining lymph nodes and spleen, reaching maximal numbers at day 10 (Figs. 4B,C). Treatment with B-MOG cells reduced this response by approximately 50%, while B-mock cells had little effect. An important function of CD4<sup>+</sup> T helper cells is to provide signals to B cells that are required for humoral immunity. Mice treated with B-MOG cells displayed markedly decreased levels of MOG-specific IgM, IgG, and IgG1 autoantibodies in serum at day 21 post-immunization, compared to untreated mice or to mice treated with B-mock cells (Fig. 4D).

The encephalitogenic function of autoreactive T cells is tightly linked to their secretion of inflammatory cytokines [19, 20]. We enumerated cytokine-producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells by flow cytometry after re-stimulation of splenocytes with MOG(35-55) for 6 hours *ex vivo* (Fig. 5). CD4<sup>+</sup> T cells expressing IFN- $\gamma$ , IL-17, and TNF- $\alpha$  transiently accumulated in spleen after immunization, reaching a peak at day 10 (Figs. 5A-C). B-MOG cells effectively suppressed these responses, and also markedly inhibited the accumulation of IFN- $\gamma$ -producing CD8<sup>+</sup> T cells (Fig. 5A-D).

### Reprogrammed quiescent B cells inhibit CNS inflammation

Clinical development of EAE is mediated by an infiltration of lymphocytes and mononuclear phagocytes in CNS. We assessed whether amelioration of clinical EAE by B-MOG cells correlated with reduced inflammation and demyelination in the CNS.

Analyses of spinal cord sections revealed that recipients of B-MOG cells had lower numbers of infiltrating T cells and macrophages compared to control mice at day 21 post EAE induction (Fig. 6A). The amount of infiltrating cells in CNS was quantified by flow cytometry at disease onset (day 10), peak of disease (day 21), and after recovery from EAE (day 35). CD45<sup>high</sup> hematopoietic cells reached maximum numbers in CNS at day 21 after EAE induction, and were reduced by about 2-fold in B-MOG cell-treated mice compared to control groups (Fig. 6B). Numbers of CD11b<sup>+</sup> myeloid cells, CD4<sup>+</sup> T cells, and CD8<sup>+</sup> T cells were also reduced in B-MOG cell-treated mice (Figs. 6C-E). The functional properties of the infiltrating T cells were determined by staining for intracellular IFN- $\gamma$  and IL-17 after re-stimulation of isolated cells with anti-CD3 and anti-CD28 for 6 hours. B-MOG cell-treated mice displayed at least a 3-fold reduction of inflammatory T<sub>H</sub>1 and T<sub>H</sub>17 CD4<sup>+</sup> T cells, as well as IFN- $\gamma$ -producing CD8<sup>+</sup> T cells, compared to control mice (Figs. 6F-H). Remarkably, B-MOG cells completely prevented the demyelinating component of the pathology in recipient mice at day 21 after EAE induction (Fig. 6A). Thus, reprogrammed

quiescent B cells markedly limited local inflammation in CNS, and prevented tissue destruction during EAE.

### Reprogrammed quiescent B cells do not influence Treg activation

B cells can regulate immunity by increasing Treg function [21, 22]. B-MOG cells restored full remission from EAE in mice lacking functional Treg cells, thus highlighting Treg-independent mechanisms of suppression (Fig. 3E). However, it remained possible that B-MOG cells promoted Treg function in wild-type mice. We therefore analyzed Treg numbers and proliferation in mice treated with B-MOG cells and in control mice at different time points after EAE induction.

B-MOG cells did not significantly increase numbers of Treg in spleen or in brain of treated mice at any time points after EAE induction (Fig. 7A). In fact, mice treated with B-MOG cells even had fewer Treg in brain at day 21, probably reflecting their milder local inflammation (Fig. 7A). It is generally assumed that the suppressive activity of Treg is related to their frequency among CD4<sup>+</sup> T cells [23]. The frequencies of Treg among CD4<sup>+</sup> T cells were similar in spleen and brain in all experimental groups of mice at different time points during EAE (Fig. 7B). We further asked whether B-MOG cells affected the proliferation of Treg by performing *in vivo* bromodeoxyuridine incorporation analyses (Fig. 7C). B-MOG cells had no effect on the percentage of proliferating Treg, which increased rapidly and reached maximum values at day 10 after immunization in spleen and brain (Fig. 7D). These data suggest that B-MOG cells regulate adaptive immune responses independently of Treg cells.

## DISCUSSION

Gene therapy is a promising approach to manipulate the immune system. Here, we show that quiescent B cells can be reprogrammed into “resting suppressor B cells” capable of inhibiting all three arms of adaptive immunity (CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and B cells) upon adoptive transfer. These B cells prevented severe disability and demyelination in EAE, and restored complete recovery from disease in mice lacking a functional Treg compartment.

Scott and colleagues pioneered the utilization of genetically modified B cells for the suppression of unwanted immunity [24]. Their approach is based on the transduction of LPS-activated B cell blasts by a retroviral vector encoding for an antigen fused to an immunoglobulin heavy chain molecule as carrier. Such B cells suppressed specific T and B cell responses upon transfer, and protected recipient mice from various autoimmune diseases [22, 24–28]. Remarkably, these B cells were uniquely able to inhibit ongoing immune responses upon administration into previously immunized recipients, while resting B cells could only prevent immune responses in naïve animals [13]. Fuchs and Matzinger also reported that LPS-activated B cells were more effective than naïve B cells at inducing tolerance [29]. Thus, mouse B cells can acquire effective suppressor functions upon appropriate stimulation *ex vivo*. Can such activation protocol be used to produce immunosuppressive human B cells? It is unknown whether the receptors eliciting suppressive functions in mouse B cells (TLR, BCR, CD40) confer similar regulatory properties to activated human B cells. Notably, TLR signaling in B cells can also promote autoimmunity, and it is important to exclude the risk of inadvertent tissue damage if activated B cells are to be injected into patients [10]. In absence of a suitable *in vitro* assay to predict the effect that activated B cells can have upon administration *in vivo*, the safety of using activated human B cells to suppress unwanted immunity in adoptive cell therapy is difficult to evaluate [9].



These concerns led us to consider resting B cells rather than activated B cells as a platform for immunosuppressive therapy. Using lentiviral vectors to deliver genes into such non-dividing hematopoietic cells [30, 31], we report here for the first time that quiescent B cells can be reprogrammed into resting suppressive B cells for adoptive cell therapy against undesirable immune responses. This approach highlights several interesting features for possible clinical application. First, the transduction protocol is simple, rapid, and highly effective, as deduced from the amounts of B cells expressing hCD4 after transduction with pDBR-hCD4. Furthermore, a protocol is already available to transduce resting human B cells with lentiviral vectors [32]. Second, mouse and human B cells maintain a quiescent phenotype upon lentiviral transduction, implying that such reprogrammed B cells should remain minimally immunogenic. The maintenance of a quiescent phenotype by the transduced B cells is remarkable given that lentiviral vectors can trigger DC maturation *in vitro* and *in vivo*, and be immunogenic upon administration into animals [33, 34]. Notably, this finding is consistent with the concept that TLR signaling is radically different in B cells compared to other populations of antigen-presenting cells [4, 35]. Third, this strategy does not rely on the assumption that signaling via TLR, CD40, or BCR is conserved between mouse and human B cells. Fourth, this approach allows the suppression of immunity towards a given antigen without requiring prior knowledge of individual epitopes and their MHC restriction, as the complete antigen coding sequence can be inserted into the lentiviral vector. This is certainly an important advantage for treating genetically diverse populations of patients. Lentiviral vectors can carry relatively large inserts up to 10 Kb, allowing the expression of a broad range of antigenic sequences [36]. Furthermore, the selected antigen can be stably expressed at high levels in resting B cells, allowing a sustained antigen presentation. Fifth, the protocol does not involve prolonged manipulation of the B cells *ex vivo* since the transduction step itself lasts only for 75 minutes, and the complete procedure of B cell isolation, engineering, and reinfusion may be as short as 5 hours. Sixth, our engineering protocol allows expression in quiescent B cells of selected molecules in addition to antigen, which may offer the possibility of strengthening the immunosuppressive functions of the antigen-presenting quiescent B cells.

We initially postulated that the protective value of B-MOG cells would be increased if these cells could additionally secrete immune modulating cytokines. However, B-MOG cells protected recipient mice from EAE independently of IL-10, as previously found in other studies on the tolerogenic functions of resting B cells [12, 37], and IL-10 expression did not augment the protective function of B-MOG cells. In contrast, IL-10 was essential to the suppressive functions of activated B cells in various experimental models [2, 4, 38]. Thus, resting and activated B cells may induce tolerance via different mechanisms, in accordance with their distinct effects on immunity upon adoptive transfer into naïve or primed recipient animals [37]. It is possible that activated B cells but not resting B cells migrate to microenvironment where IL-10 can mediate regulatory functions. Alternatively, IL-10 may act in an autocrine manner on the activated B cells themselves to modulate their co-stimulatory functions and confer them with suppressive properties, while this cytokine may have little effect on the expression of co-stimulatory molecules by resting B cells. It will be essential to investigate the mechanisms involved in the suppressive functions of resting and activated B cells in order to develop novel generations of “resting regulatory B cells” with improved therapeutic potentialities. Resting transgenic B cells expressing MOG(35-55) antigen fused to the invariant chain Ii stimulated an increased expression of CTLA-4 and PD-1 on antigen-reactive CD4<sup>+</sup> T cells, thereby programming these cells to enter apoptosis upon subsequent antigenic stimulation, as elegantly shown by Frommer and colleagues [12]. Further studies will be required to evaluate whether similar mechanisms underlie the suppressive functions of B-MOG cells, and of the resting transgenic B cells used by Frommer and colleagues. In support of this possibility, the two types of B cells were similarly capable of protecting recipient mice independently of Tregs, even though they

were engineered in different ways. Given their Treg-independent function, these B cells may be beneficial to treat MS patients, whose Treg activity is compromised [17, 39]. In particular, administration of antigen-expressing quiescent B cells may be a useful means of preventing recruitment via epitope spreading of novel autoreactive T and B cell clones into the pathogenic process, which plays an important role in progression of autoimmune diseases [40]. For instance, resting B cells presenting different myelin antigens could be administered to relapsing-remitting MS patients in remission in order to limit further relapse. It remains open whether resting B cells could be engineered in a way enabling them to intercept disease when injected at the time of a relapse. Currently, treatment of RR-MS relapses is done with corticosteroids [41]. B-MOG-IL-10 cells did not show any protective effect when administered to mice at peak of EAE (data not shown). Further studies will investigate whether B-MOG cells engineered to express molecules involved in killing of activated T cells such as FasL, or agonists of CTLA-4 (which is important for control of ongoing immunity [13, 37]) might be able to accelerate remission from disease when administered during an acute EAE flare.

Beyond autoimmunity, reprogrammed resting B cells could be of interest to prevent immune reactions towards therapeutic proteins such as factor VIII, which is used to treat hemophilia A, or to therapeutic enzymes, which are used to treat patients with lysosomal storage diseases [42, 43]. Similarly, they could be used to avoid the elimination of gene-modified cells by the immune system after gene therapy, a process that can diminish the long-term efficacy of this therapeutic approach [44]. Importantly, gene therapy products and protein therapeutics usually represent neo-antigens for the recipient's immune system, which is therefore not tolerant. Furthermore, they are not expressed in the thymus, so it is unlikely that mechanisms of dominant tolerance involving Treg limit immunity to these therapeutic molecules. On this point, it is directly relevant that quiescent re-programmed B cells can suppress immunity independently of Treg, because they should therefore be able to effectively suppress immunity to neo-epitopes contained in therapeutic proteins or gene therapy products.

There is increasing interest in using lentiviral vectors for gene therapy. The evidence gained from the first clinical trials that such approach is safe, together with the development of new systems such as non-integrative vectors, should further stimulate their utilization in treating human diseases [45, 46]. Here, we described a novel potential application of this technology, that is, the prevention of unwanted immunity using genetically engineered resting regulatory B cells.

## MATERIALS AND METHODS

### Mice and immunization

C57BL/6 and IL-10<sup>-/-</sup> mice were bred at Bundesinstitut für Risikobewertung (Berlin, Germany), at the University of Edinburgh (Edinburgh, UK), at Université Paris-Sud (Orsay, France), and treated according to French, German, and U.K. legislations. EAE was induced with MOG(35-55) as described [2].

### Lentiviral vectors and production of viral supernatants

pDBR was produced by extensive alteration of pLL3.7 [47] i.e. removal of U6-shRNA and GFP cassettes by XbaI/XhoI and NheI/EcoRI digestions (pDBR sequence is available at EMBL/ENA (accession number FR822201), and is provided in supplementary Fig. 1). A multiple cloning site was introduced (Fig. 1). Mouse *Mog*, *Il-10*, and *hCd4* were amplified using the primer pairs 5'-ATGGATCCCGCCACCATGGCCTGTTTGTGGAGC-3' and 5'-ATCTCGAGACTCAAAAGGGGTTTCTTAGC-3', 5'-



ATGGATCCCGCCACCATGCCTGGCTCAGCACTGC-3' and 5'-  
 ATCTCGAGCACACTGCAGGTGTTTTAGC-3', 5'-  
 ATTCTAGAGCCACCATGTGCCGAGCCATCTCTCT-3' and 5'-  
 ATCTCGAGTCTAGTGCCGGCACCTGACACAGC-3', and cloned into pDBR using  
 BamHI/XhoI or XbaI/XhoI. Lentiviral particles were produced in 293T cells by co-  
 transfection of psPax2 packaging vector (Addgene), YITG envelope plasmid (kindly  
 provided by Ken Murphy), and pDBR plasmid using a calcium phosphate method. After 48  
 h, supernatants were collected, filtered (0.45  $\mu$ m), and supplemented with HEPES buffer  
 (Gibco-Invitrogen) at 10 mM and hexadimethrine bromide at 10  $\mu$ g/ml (Sigma-Aldrich).

### B cell transduction

B cells isolated from spleen using anti-CD43 microbeads (Miltenyi Biotec) (>98% pure) were centrifuged at  $3.5 \times 10^6$ /well in 6-well plates with 3 ml of viral supernatants in a total volume of 4 ml at 1800 rpm during 75 min at room temperature, and then washed in PBS. Some cells were administered immediately into mice, and others were kept in culture in RPMI medium+Glutamax (Gibco-Invitrogen) supplemented with 10% FCS, Penicilline/Streptomycine (PAA) and 5-Mercaptoethanol (PAA), and confirm secretion of IL-10, and presentation of MOG.

### Antigen-presentation assay

Transduced B cells were cultivated in complete RPMI medium in flat-bottom 96 well-plates with  $1 \times 10^5$  P32.2 cells. Some wells were supplemented with MOG(35-55) at 20  $\mu$ g/ml, or anti-MHC-II blocking antibody (M5/114) at 30  $\mu$ g/ml. Supernatants were harvested after 48 h, and IL-2 was measured by ELISA.

### ELISA

Microtiter plates (Costar) were coated with anti-IL-10 (SXC1), -IL-6 (20/F3), -IL-2 (JES6-1A12). Detection was performed with biotin-anti-IL-10 (JES5-2A5-7), -IL-6 (MP5-32C11) or -IL-2 (JES6-5H4). For anti-MOG antibody ELISA, plates were coated with MOG(35-55) at 20  $\mu$ g/ml.

### Bone marrow-derived macrophages

Bone marrow cells were plated in 100 mm culture dishes (CELLSTAR, Greiner Bio-One). Non-adherent cells were harvested after 16 h, and transferred to 100 mm Petri dishes (Greiner Bio-One) in DMEM with M-CSF. Non-adherent cells were removed on days 3 and 6. Macrophages were harvested on day 7, plated at  $1.25 \times 10^5$ /well in 96-well flat bottom plate, and activated with 1  $\mu$ g/ml CpG (ODN 1826, TIB MOLBIOL) for 24 h. Some wells were pre-incubated for 30 min with 100  $\mu$ l of B cell supernatants, or mouse IL-10 (20 ng/ml; BD Pharmingen) before addition of CpG. TNF- $\alpha$  was quantified using the anti-TNF- $\alpha$  ELISA *ready set-go* kit (eBioscience).

### Preparation of mononuclear cells from CNS

Brains were removed from perfused mice and digested with collagenase (Worthington Biochemical) and deoxyribonuclease (Sigma-Aldrich) followed by mechanical disaggregation. Mononuclear cells were isolated on a 30% Percoll gradient (GE Healthcare) by centrifugation for 20 min at 2000 rpm at room temperature.

### Flow cytometry

Following blocking of Fc receptors (2.4G2), stainings were done with antibodies against CD19 (1D3), hCD4 (TT1), CD4 (GK1.5), CD8 $\alpha$  (53-6.7), CD11b (M1/70), MHCII (M5/114), CD40L (MR1), CD80 (16-10A1), CD86 (GL-1), CD40 (3/23), CD44 (IM7),

CD69 (H1.2F3), IFN- $\gamma$  (XMG1.2), TNF- $\alpha$  (MP6-XT22), IL-17 (TC11-18H10), Foxp3 (FJK-16a), BrdU (B44). Antibodies were from BD Pharmingen (San Diego, CA), eBioscience (San Diego, CA), Miltenyi Biotec (Bergisch Gladbach), or produced in house. Dead cells were excluded with propidium iodide (PI). After transduction, B cells were seeded at  $2.5 \times 10^6$ /well in 48-well plates, and analyzed 18 h later for expression of activation markers, while supernatants were characterized by ELISA. Alternatively,  $1 \times 10^7$  transduced B-hCD4 cells were injected into C57BL/6 mice. Recipient mice were sacrificed 18 h later, spleens were harvested and expression of activation markers was analysed on B-hCD4 positive cells. LPS-activated B cells were cultured at similar density with 5  $\mu$ g/ml LPS (*E.coli* 055:B5, Sigma). For intracellular staining, splenocytes were seeded at  $4 \times 10^6$ /well in flat-bottom 48-well plates, and re-stimulated with MOG(35-55) (20  $\mu$ g/ml) in presence of Golgistop as recommended (BD Pharmingen). After 6 h, surface and intracellular cytokine stainings were done using the Cytofix/Cytoperm kit (BD biosciences). Brain-infiltrating mononuclear cells were seeded as described, and stimulated with anti-CD3+anti-CD28 (145-2C11 at 0,1  $\mu$ g/ml, and 7D4 at 1  $\mu$ g/ml, BD Pharmingen).

### Histopathology

2-3  $\mu$ m sections of formalin-fixed, paraffin-embedded spinal cord tissue were deparaffinized, and subjected to heat-induced epitope retrieval. T cells and macrophages were stained with polyclonal rabbit antibodies against CD3 (Dako) or Iba-1 (Wako), respectively, followed by detection with biotinylated donkey anti-rabbit antibody (Dianova) and streptavidinAP kit using the Fast Red chromogen (Dako). Luxol fast blue/ Periodic-Acid-Schiff (LFB-PAS) stainings were performed according to standard procedures.

### Foxp3 and BrdU staining

Mice received 1 mg BrdU intra-peritoneally 12 h before analysis. BrdU was detected with the BrdU Flow Kit (BD Pharmingen) using the fix/perm buffer from the eBioscience Foxp3 staining kit. Data were acquired on FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc.).

### Statistical analyses

All statistical analyses were performed with GraphPad Prism 4 using t-test or ANOVA tests (GraphPad Software Inc.). \*,  $P < 0.05$ . \*\*,  $P < 0.01$ . \*\*\*,  $P < 0.0001$ .

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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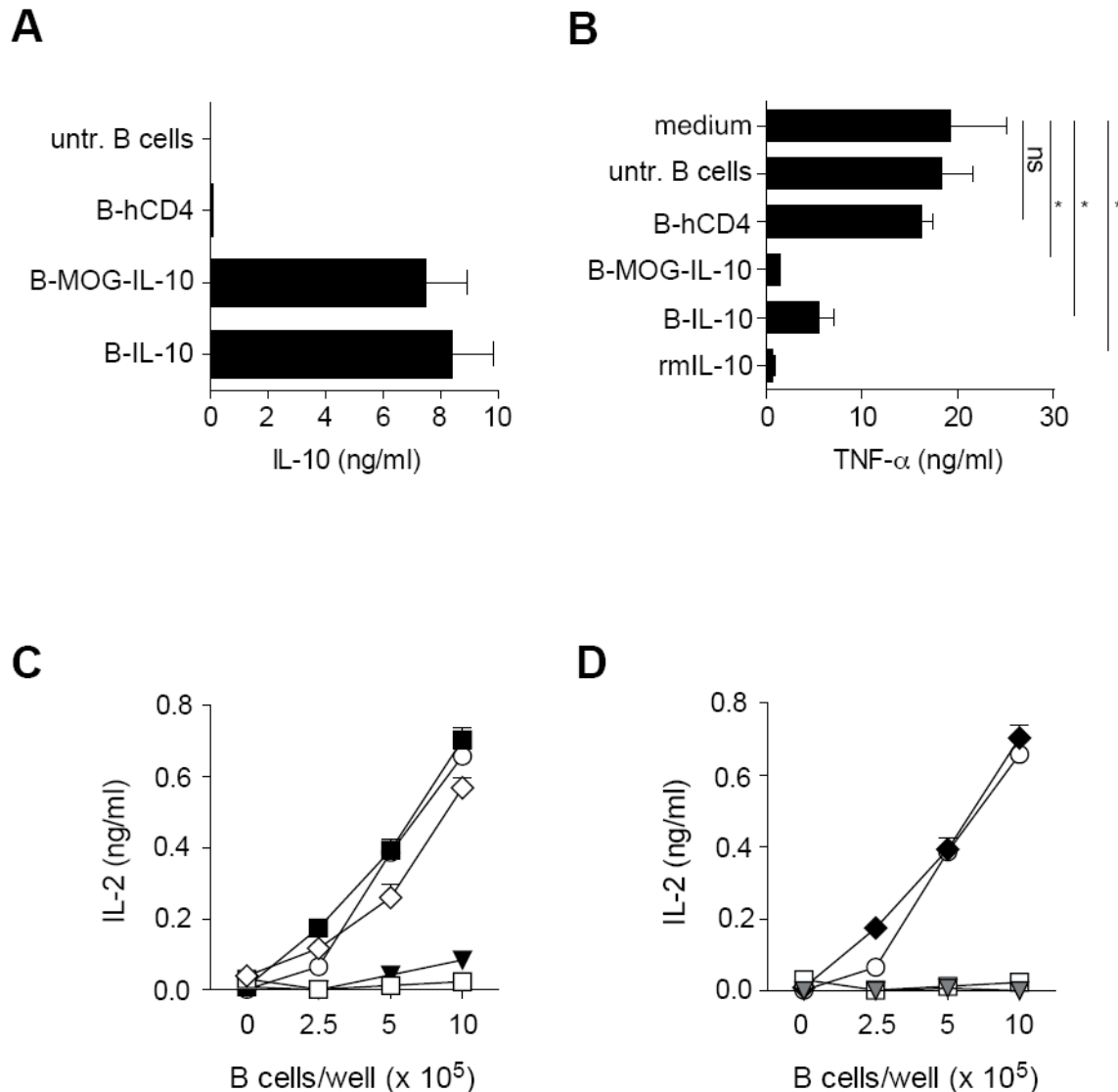
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## Abbreviations

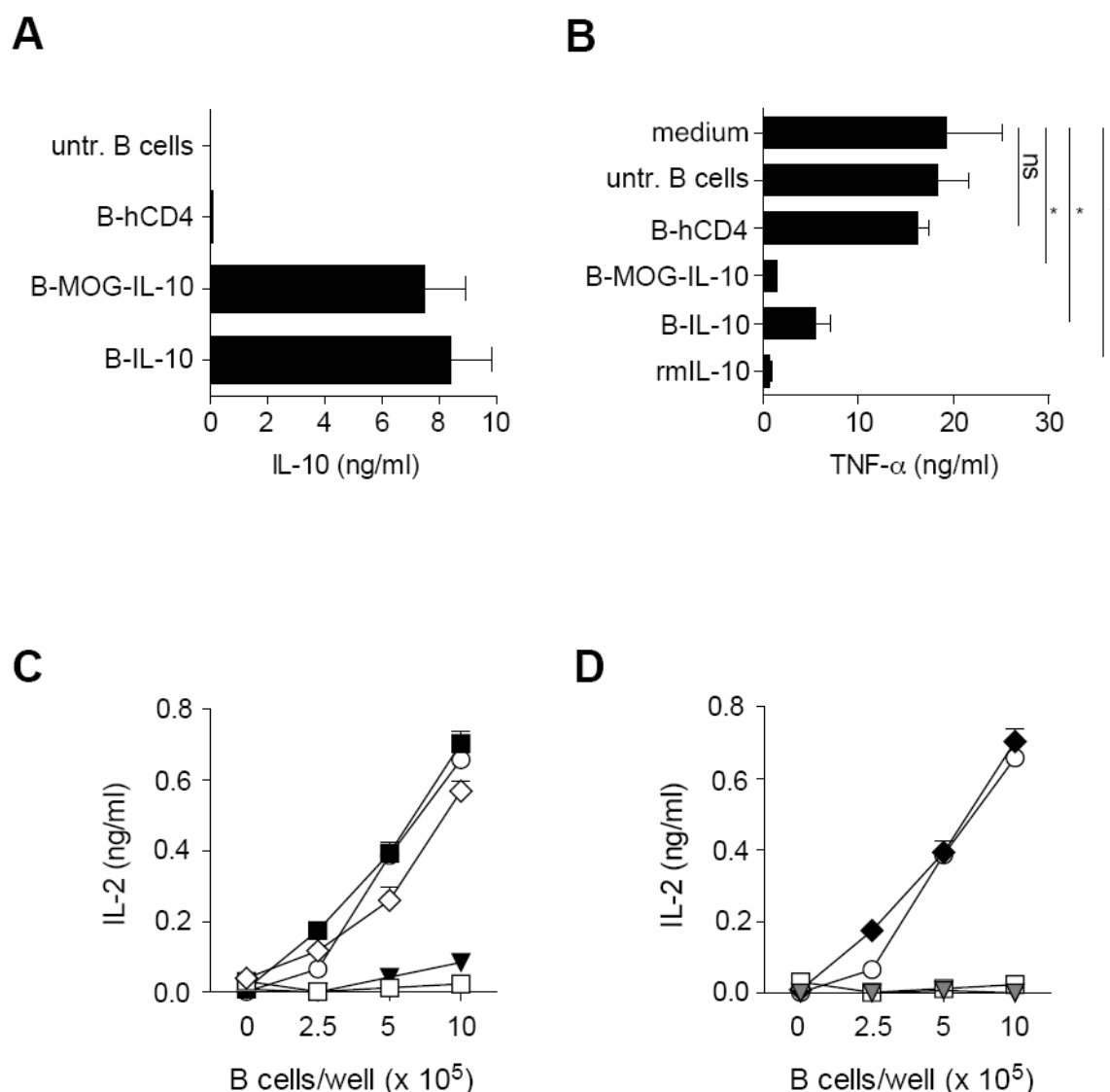
**MOG**      myelin oligodendrocyte glycoprotein



**Figure 1. Effective transduction of resting mouse B cells with an HIV-based lentiviral vector**  
 (A) Schematic representation of the pDBR vector. Isolated B cells were transduced with pDBR-hCD4, or left untransduced, and analyzed 18 h later for surface expression of hCD4. Histogram plot shows intensity of hCD4 surface expression on transduced B cells (solid line) and untransduced B cells (dotted line). (B) B cells were left untransduced (untr. B cells; dotted line), transduced with pDBR-hCD4 (B-hCD4 cells; black line), or left untransduced and activated with 5  $\mu$ g/ml LPS (LPS; grey line). Cells were analyzed by flow cytometry for surface expression levels of MHC-II, CD80, CD86, CD40, CD69, and CD44 after 18 h. Analyses were done after gating on live CD19<sup>+</sup>PI<sup>-</sup> cells, except for B-hCD4 in which case the gating was on CD19<sup>+</sup>PI<sup>-</sup>hCD4<sup>+</sup>. Histograms show representative stainings of 3 independent experiments. (C) Mean fluorescence intensity (MFI) for the stainings described in (B), compiling the data from the 3 independent experiments. (D) Amount of IL-6 in culture supernatants from the three types of treated B cells at 18 h post-transduction. Data are compiled from 4 independent experiments. (E) B cells transduced with pDBR-hCD4 were injected intravenously into C57BL/6 mice. Recipient mice were analyzed 18 h later to determine expression levels of the activation markers on B-hCD4 cells present in spleen.

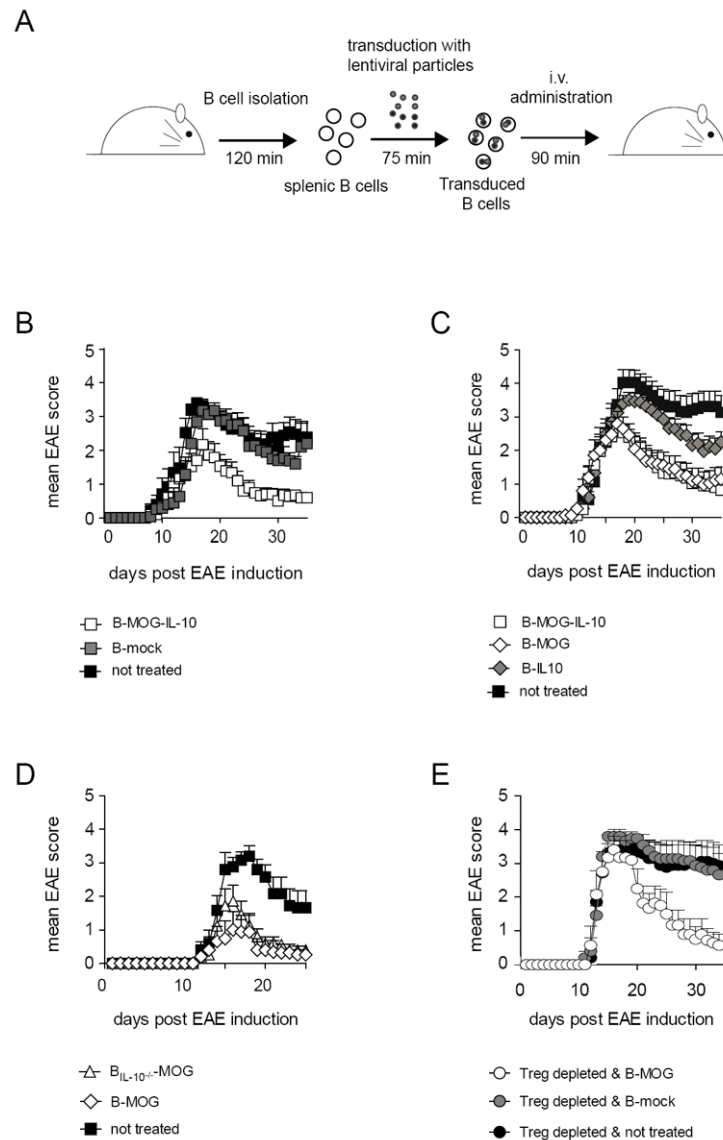


Data are compiled from two independent experiments). Results are expressed as mean  $\pm$ s.e.m., \*,  $P<0.05$ . \*\*,  $P<0.01$ . \*\*\*,  $P<0.0001$



**Figure 2. Engineering of IL-10-secreting antigen-presenting quiescent B cells**

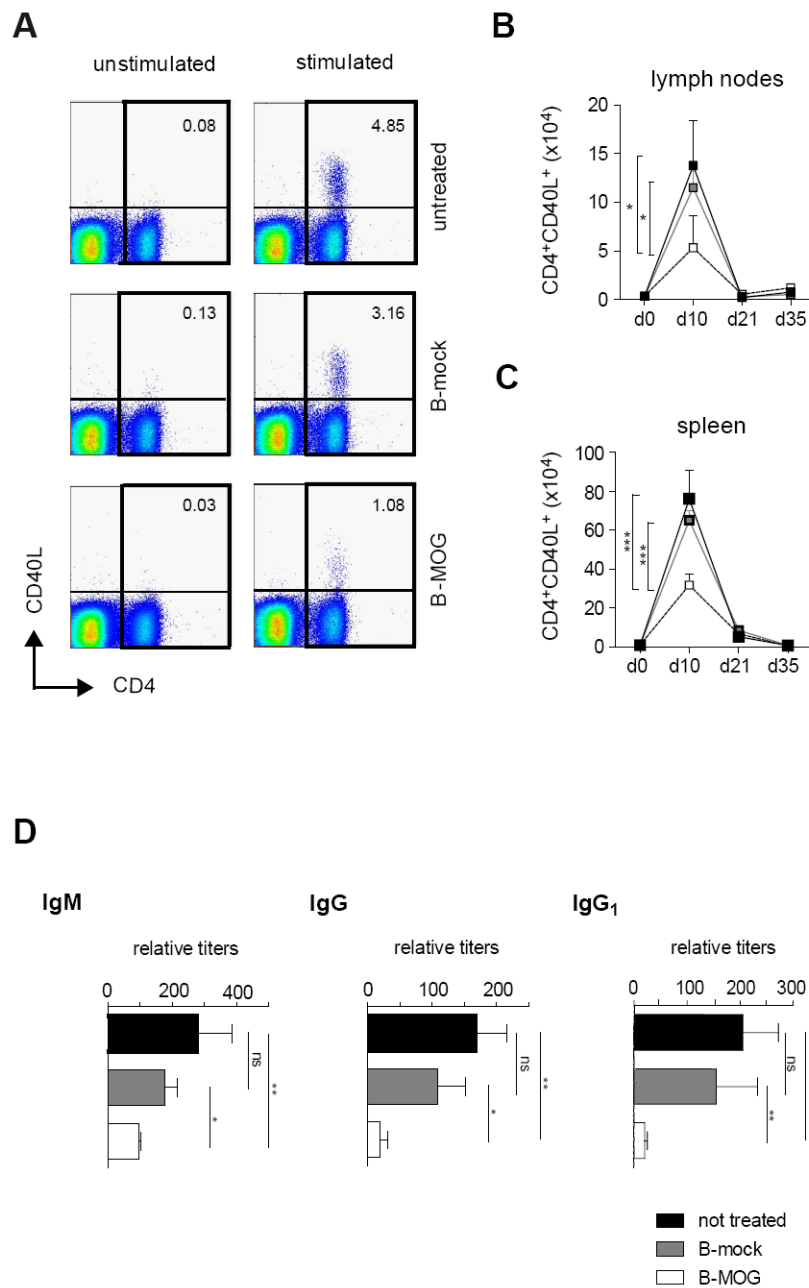
(A) B cells were transduced with pDBR-hCD4 (B-hCD4 cells), or pDBR-MOG and pDBR-IL-10 (B-MOG-IL-10 cells), or pDBR-IL-10 (B-IL-10 cells), and IL-10 was quantified in culture supernatants at 24 h post-transduction (n=8). (B) B cells were transduced as described in (A), and the culture supernatants were collected at 18 h post-transduction. These supernatants were then added to macrophages to measure their effect on TNF- $\alpha$  secretion induced by CpG activation. Recombinant mouse IL-10 (rmIL-10) was used as positive control. (n=4). (C) The MOG-reactive T cell hybridoma P32.2 ( $1 \times 10^5$  cells) was stimulated with 2-fold increasing numbers of untransduced B cells (white squares), untransduced B cells pulsed with MOG(35-55) (black squares), B-MOG cells (white circles), B-MOG-IL-10 cells (white diamonds), or B-mock cells (black triangles). IL-2 production was measured after 48 h (n=4). (D) P32.2 cells were cultured as in (C) with untransduced B cells (white squares), untransduced B cells pulsed with MOG(35-55) (black diamonds), B-MOG cells (white circles), or both B-MOG cells and an anti-MHC-II blocking antibody (grey triangles). IL-2 production was measured after 48 h (n=4). Results are expressed as mean  $\pm$  s.e.m. Each experiment was repeated at least 3 times. \*,  $P < 0.05$ .



### Figure 3. Engineered resting B cells provide protection from chronic EAE

(A) Schematic representation of the B cell-based therapeutic protocol. (B) Mice were treated intravenously with  $1 \times 10^7$  B-MOG-IL-10 cells (white squares), or B-mock cells (grey squares) on days -6 and -2 before EAE induction, or left untreated (black squares). Mice were then assessed daily for EAE disease scores.  $p < 0.01$  for cumulative disease scores of B-MOG-IL-10 treated mice as compared with B-mock-treated mice or untreated mice. (C) Mice were treated with B-MOG-IL-10 cells (white squares), or B-MOG cells (white diamonds), or B-IL-10 cells (grey diamonds) on days -6 and -2 before EAE induction, or left untreated (black squares).  $p < 0.01$  for cumulative disease comparing B-MOG and B-MOG-IL-10-treated mice with untreated mice. (D) Mice were treated with IL-10-deficient B cells transduced with pDBR-MOG ( $B_{IL-10^{-/-}}$ -MOG) (white triangles), or B-MOG cells (white diamonds) on days -6 and -2 before EAE induction, or left untreated (black squares).  $p < 0.05$  for cumulative disease of B-MOG- and  $B_{IL-10^{-/-}}$ -MOG-treated mice compared with untreated mice. (E) Mice received an anti-CD25 antibody (PC61) intravenously to inactivate natural  $CD4^+CD25^+$  T regulatory cells on day -3. Mice were additionally treated with B-MOG cells (white circles), or B-mock cells (grey circles) on days -6 and -2 before EAE

induction, or left untreated (black circles).  $p < 0.05$  for the cumulative disease of B-MOG-treated mice compared with untreated mice. Each experiment comprised of 5 mice per group, and was repeated at least 3 times. Data shown are mean EAE scores  $\pm$  s.e.m.

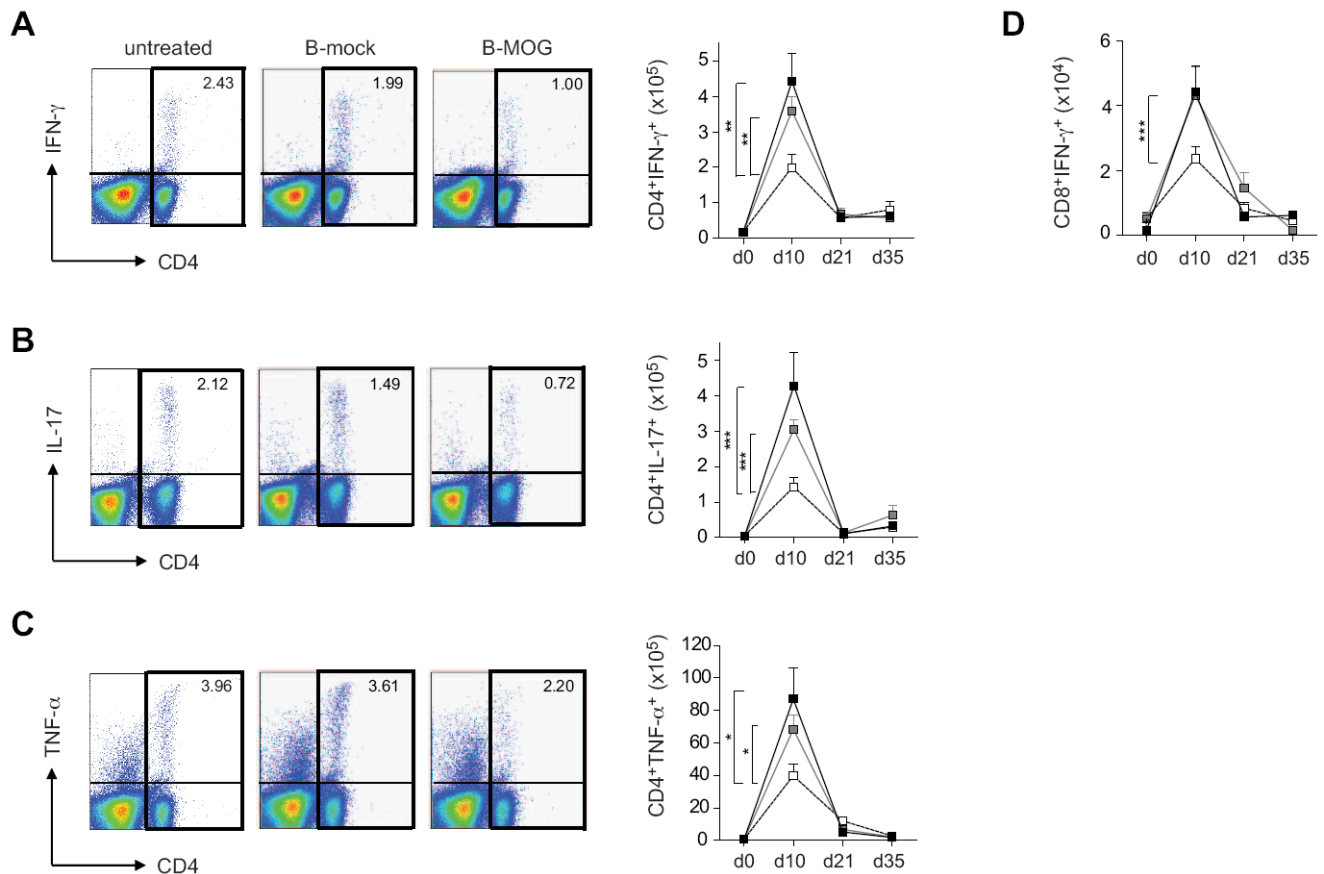


**Figure 4. Engineered resting B cells suppress T and B cell responses in secondary lymphoid organs**

Mice were treated on days -6 and -2 with B-MOG cells, or B-mock cells, or left untreated, and analyzed at different time points after EAE induction. Cells were isolated from spleens and draining lymph nodes, and either re-stimulated with MOG(35-55) for 6 h *ex vivo*, or left unstimulated as controls. (A) Representative stainings for surface CD4 and intracellular CD40L for splenocytes from mice at day 10 after EAE induction. Numbers indicate frequencies of CD40L<sup>+</sup> among CD4<sup>+</sup> T cells. (B, C) Numbers of CD4<sup>+</sup>CD40L<sup>+</sup> T cells calculated from these frequencies in (B) draining lymph nodes and (C) spleen of mice treated with B-MOG cells (white squares), or B-mock cells (grey squares), or untreated cells (black squares) analyzed at the indicated time points. (D) Relative titers of MOG(35-55)-

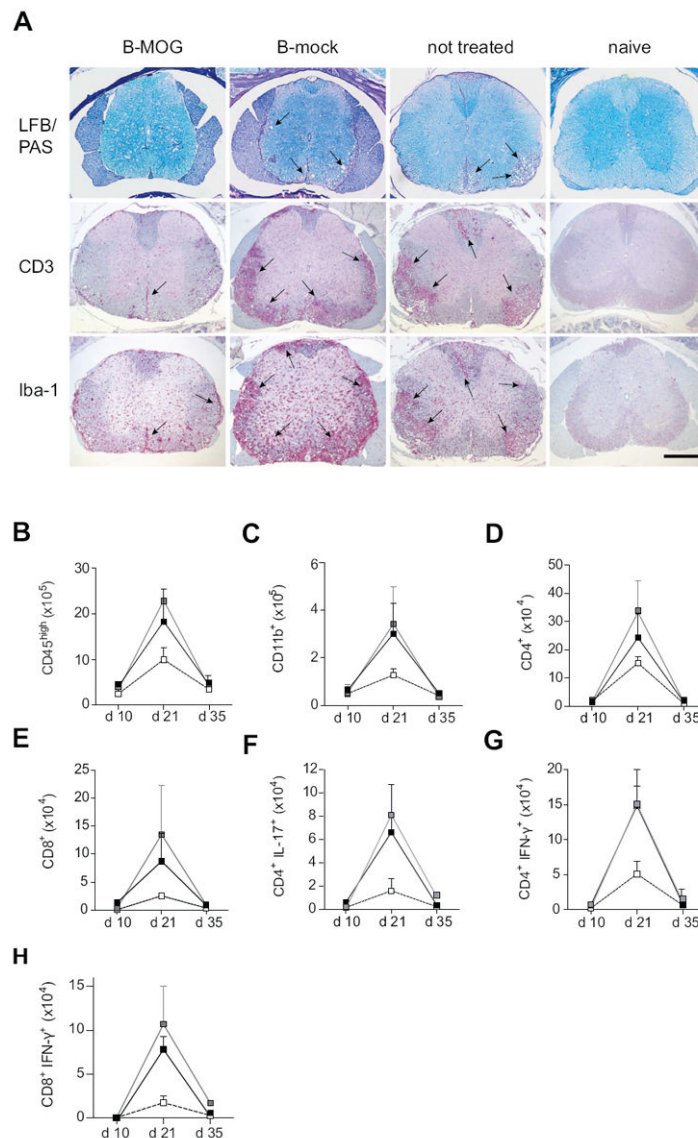
reactive IgM, IgG and IgG<sub>1</sub> in serum at day 21 after EAE induction were determined by ELISA. Each experiment comprised 4-5 mice per group, and was repeated 3 times. Results are expressed as mean±s.e.m. \*, P<0.05. \*\*, P<0.01. \*\*\*, P<0.0001





**Figure 5. Engineered resting B cells suppress inflammatory T cell responses of TH1 and TH17 types in spleen**

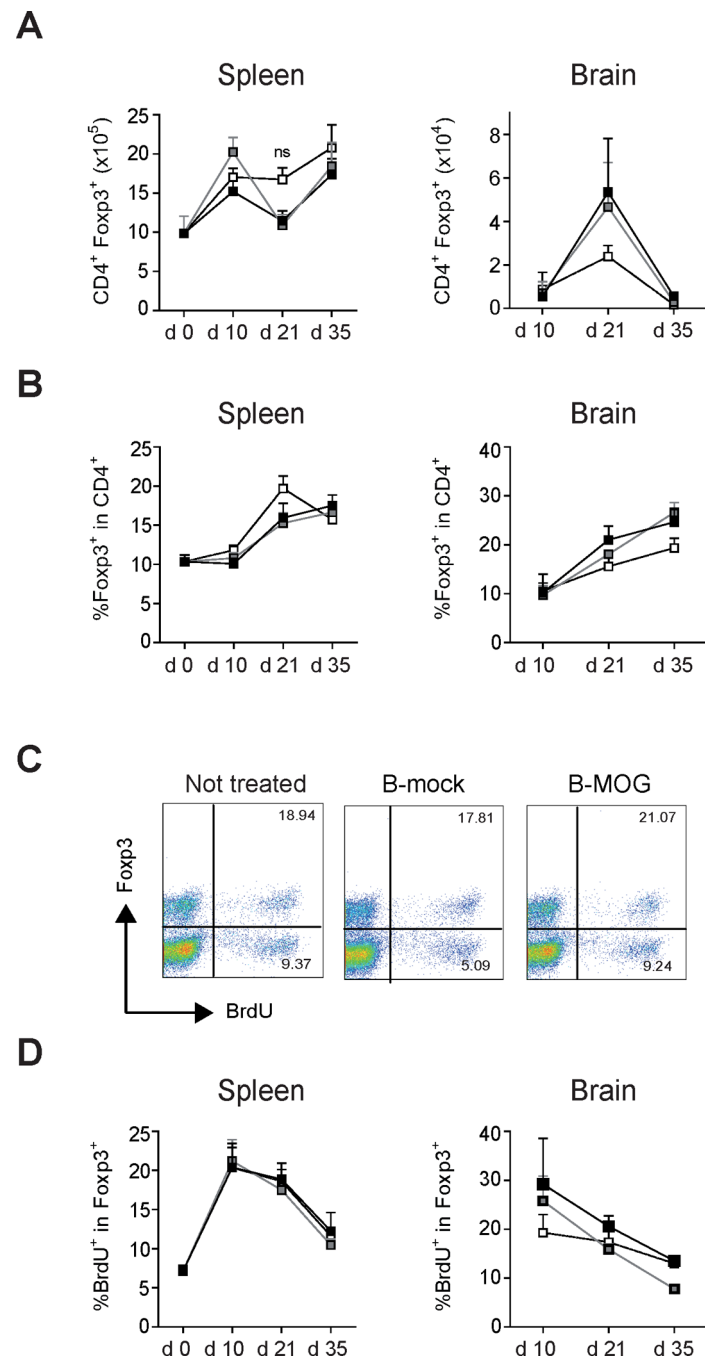
Splenocytes from mice treated on days -6 and -2 with B-MOG cells, B-mock cells, or from untreated mice were taken at different time points after EAE induction, and re-stimulated with MOG(35-55) for 6 h to measure production of cytokines by CD4<sup>+</sup> and CD8<sup>+</sup> T cells by flow cytometry. (A-C) Representative intracellular stainings for IFN- $\gamma$ , IL-17, and TNF- $\alpha$  in CD4<sup>+</sup> T cells taken from mice at day 10 after EAE induction and re-stimulated for 6 h with MOG(35-55) *ex vivo*. Frequencies of cytokine-positive cells among CD4<sup>+</sup> T cells are indicated. (A-D) Numbers of (A) CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup>, (B) CD4<sup>+</sup>IL-17<sup>+</sup>, (C) CD4<sup>+</sup>TNF- $\alpha$ <sup>+</sup>, and (D) CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells from mice treated with B-MOG cells (white squares) or B-mock cells (grey squares), or untreated mice (black squares). Each experiment was repeated at least 3 times with 4-5 mice per group per time point. Results are expressed as mean $\pm$ s.e.m. \*, P<0.05. \*\*, P<0.01. \*\*\*, P<0.0001



**Figure 6. Engineered resting B cells protect recipient mice from CNS infiltration and demyelination**

(A) Spinal cords from mice treated on days -6 and -2 with B-MOG cells, or B-mock cells, or from untreated mice were collected on day 21 after EAE induction. Sections were stained for myelination (luxol fast blue/ Periodic-Acid-Schiff), T cells (CD3), and macrophages (Iba-1). Spinal cord sections from naïve mice were used as controls. Arrows show demyelinated areas (luxol fast blue/Periodic-Acid-Schiff staining), infiltrated T cells (CD3 staining), and infiltrated macrophages (Iba-1 staining). A representative picture from sections of a mouse per group is shown. Scale bar: 500  $\mu$ m. (B-H) Mononuclear cells were isolated from brains of mice treated on days -6 and -2 with B-MOG cells (white squares), or B-mock cells (grey squares), or from untreated mice (black squares) at the indicated time points after EAE induction. Numbers of (B) CD45<sup>high</sup> cells, (C) CD11b<sup>+</sup> myeloid cells, (D) CD4<sup>+</sup> T cells and (E) CD8<sup>+</sup> T cells per brain. (F, G, H) Cells were stimulated *ex vivo* with anti-CD3+anti-CD28 antibodies for 6 h, and cytokine production was quantified in CD4<sup>+</sup> and CD8<sup>+</sup> T cells by intracellular staining and flow cytometry. Number of (F) CD4<sup>+</sup>IL-17<sup>+</sup> T cells, (G) CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells, and (H) CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells per brain. Each experiment

comprised 4-5 mice per group per time point, and each analysis was repeated at least 3 times. Results are expressed as mean $\pm$ s.e.m.



**Figure 7. Engineered resting B cells do not affect natural Treg during EAE**

Mice were treated on days -6 and -2 with B-MOG cells (white squares), or B-mock cells (grey squares), or were left untreated (black squares), and analyzed at the indicated time points after EAE induction. Treg cells were characterized by staining for CD4 and Foxp3. (A) Number of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells per spleen or brain. (B) Frequency of natural Foxp3<sup>+</sup> Treg cells among CD4<sup>+</sup> T cells in spleen or brain. (C, D) Mice were administered with 1 mg BrdU 12 h before analysis to characterize proliferative activity of Treg cells. (C) Representative stainings gated on CD4<sup>+</sup> T cells for Fop3 and BrdU. (D) Frequency of proliferating BrdU<sup>+</sup> cells among CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells in spleen or brain. Each

experiment comprised 4-5 mice per group per time point, and was repeated at least 2 times. Results are expressed as mean $\pm$ s.e.m.